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NOTICES

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Editor : N. A. Marris, M.Sc., B.Com. (Assistant Editor : M. O'Connor, M.Sc.)
Department of Scientific and Industrial Research, Wellington

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THE METABOLISM AND TOXICITY OF CYANIDES AND CYANOGENETIC GLUCOSIDES IN SHEEP:
II. DETOXICATION OF HYDROCYANIC ACID

By R. L. BLAKLEY and I. E. COOP

(*Received for publication, 23 September 1948*)

Summary

1. It is unlikely that HCN reacts chemically in the rumen of sheep to any appreciable extent, except possibly in cases when sulphur compounds are administered per os.

2. In sheep and humans only very small amounts of administered KCN appear as HCN or cyanide in urine breath, and saliva.

3. Blood concentrations of cyanide in sheep after dosing with KCN are low, receding from a maximum at about 15 min. after dosing to zero after about 5 hours. Serum thiocyanate reaches much higher concentrations and does not return to normal until 24-48 hours after dosing.

4. Thiocyanate formation from cystine and HCN in presence of sheep liver extracts has been studied, and it is suggested that it may proceed through intermediate formation of α amino $-\beta$ thiocyanopropionic acid.

Thiocyanate synthesis from HCN in presence of liver extract occurs much more rapidly utilizing sulphide or thiosulphate in place of cystine. The presence of H_2S in the rumen and its rapid absorption suggest that it is probably the most important sulphur donor.

5. Thiocyanate is excreted into the urine slowly over a period of several days. Recoveries of over 50 per cent have been obtained indicating that thiocyanate formation is the most important though not necessarily the only mode of detoxication.

6. No evidence is found for cyanhydrin formation by glucose with HCN in presence of serum or liver extract.

IN the previous paper it has been shown that under most conditions which are likely to obtain in practice, hydrocyanic acid derived from potassium cyanide or from cyanogenetic glucosides is rapidly absorbed from the rumen of sheep. The toxicity of cyanide under various conditions of dosing and the danger of cyanogenetic plants to stock must therefore depend largely on the nature and capacity of the detoxication system of the animal. Moreover the efficacy of various antidotes for cyanide poisoning and their mode of action must depend on the mechanism of detoxication. The present investigation was therefore undertaken to provide more information on the detoxication system in sheep.

Extensive evidence has been presented to show that in many animals cyanide is mainly converted to thiocyanate which appears in the urine. Mukerji and Smith (1) found that a large proportion of cyanide administered parenterally to the rabbit in sublethal doses was recovered in the urine as thiocyanate within 24-48 hours. In the dog, excretion of thiocyanate was much slower and only a small proportion of the cyanide was recovered as thiocyanate. Similar results for the rabbit were obtained by Baumann, Sprinson and Metzger (2), who were able to recover 75 per cent of injected cyanide as thiocyanate. Meyer and Bodansky (3) administered orally 10mg. doses of potassium cyanide to human patients and were able to recover up to 10 per cent as thiocyanate in saliva samples taken during the three hours following the dose.

Further evidence for thiocyanate formation is provided by the fact that sulphur compounds act as antidotes for cyanide poisoning. It is widely recognized that thiosulphate will protect against several times the lethal dose and Forst (4) found that injection of colloidal sulphur was even more effective. Tetrathionate has also been reported as possessing some antidotal properties. Voegtlin, Johnson and Dyer (5) found that cystine, cysteine, glututhione and thioglycollic acid injected intravenously into rats all prevented death from cyanide (1.5 M.L.D.) which had been injected subcutaneously one minute previously. They found that in terms of available sulphur thiosulphate is only slightly more effective than cystine or cysteine.

Lang (6) demonstrated rapid conversion of cyanide to thiocyanate in presence of colloidal sulphur or thiosulphate by liver extracts. He showed the reaction to be enzymic and named the enzyme responsible rhodanase. The presence of rhodanase was demonstrated in most organs although the amount varied greatly, and the enzyme was shown to be present in most animals. According to Lang direct production of thiocyanate from sulphides is not possible and he concluded that thiosulphate or elementary sulphur are the sulphur donors in cyanide detoxication *in vivo*.

Several workers have also reported the effectiveness as cyanide antidotes of aldehydes, ketones, and reducing sugars which they assumed to act by forming cyanhydrins but more direct evidence of cyanhydrin formation has proved difficult to obtain. It has been shown, however, that compounds of this type inhibit the hæmolytic effect of potassium cyanide on red corpuscles *in vitro* (7), inhibit the action of potassium cyanide on the respiration of liver tissue (8), and on bird erythrocytes (9), and remove the poisoning effect of hydrocyanic acid on the heart (9). Thomas and Bigwood (10) showed that the variation with pH of the reducing power and optical rotation of dialysates of the blood of rabbits or dogs is almost completely eliminated by addition of hydrocyanic acid. They attributed this effect to cyanhydrin formation with blood glucose. Potassium cyanide loses its toxic effect for pigeons when incubated with a reducing sugar at 37°C. for 24 hours (11).

In the present studies the modes of excretion of cyanide and its detoxication products have been investigated in the sheep and the thiocyanate reaction further studied.

METHODS OF ANALYSIS

(i) *Thiocyanate* in serum and solutions containing tissue extract was determined by the method of Aldridge (12). The sample was run

into a solution containing tungstic acid, mixed thoroughly and allowed to stand for at least 10 min. to complete precipitation. After centrifuging, 2 ml. of the supernatant was used for the estimation. When the sample contained buffer it was sometimes necessary to add 1-2 drops of concentrated H_2SO_4 to complete precipitation. The colour was compared in a Duboscq colorimeter with that of a standard, prepared by treating in the same way standard thiocyanate of similar strength to the unknown. Thiocyanate added to serum could be recovered with an accuracy of $\pm$ 5 per cent and close agreement was obtained between values of serum thiocyanate obtained by this method and those determined using ferric reagent with serum deproteinated with trichloracetic acid. To determine thiocyanate in presence of cyanide, the deproteinated solution was first aerated for 5 min. at 80-90°C., dilution due to washing out of the aeration tube being noted. Tests showed that the thiocyanate was unaffected by this treatment but cyanide was completely removed.

(ii) Attempts to apply the above method to determination of *urine thiocyanate* were unsuccessful. Urine pigments could be removed by addition to urine, and re-precipitation, of serum protein, obtained by dissolving up the tungstic acid precipitate from serum with excess Na_2WO_4. There always remained in solution, however, compounds which caused a brown colour to be developed instead of the normal red. Oxidation of urine thiocyanate to cyanide on a micro scale by persulphate, followed by estimation of the cyanide by the method of Gettler and Goldbaum (13), was also unsatisfactory. Finally the complete method of Baumann, Sprinson, and Metzger (14) was adopted.

(iii) *Cyanide* was estimated by the method of Gettler and Goldbaum with the modifications described in Part I of this series, or by the method of Aldridge after deproteinating with tungstic acid.

(iv) *H_2S* was estimated by a modification of the method adopted for determining HCN. In the Gettler and Goldbaum method of estimating HCN it was found that H_2S produces a black stain of ferrous sulphide on the paper, this stain being removed in the subsequent acid treatment. Tests showed that the stain was roughly proportional to the amount of H_2S aerated and could therefore be used as a method of estimation. As the stains faded and became more difficult to read after a few hours, fresh standards were made each time unknowns were measured. The method determines H_2S in amounts of the order 1-10 μg. with an accuracy of about $\pm$ 20 per cent.

Reaction of Hydrocyanic Acid in the Rumen

In the previous paper it was assumed that the decrease in the HCN concentration in the rumen was due solely to absorption through the wall of the rumen. However, it is probable that at least a small portion of the HCN is converted to thiocyanate in the rumen. Lang (6) demonstrated a slow synthesis of thiocyanate by B. coli commune from HCN and thiosulphate, and Loffler and Rigler (15) have shown that bacteria that decompose cystine with evolution of H_2S and production of sulphur exhibit an elevated tolerance for HCN. H_2S was frequently detected in considerable amounts (1–5 μg./ml.) during analysis of ruminal fluid for HCN. Hence under normal conditions the ruminal microflora would be expected to convert some of the cyanide to thiocyanate.

In the first experiments KCN was incubated at 37°C. in Conway units with fresh ruminal fluid (pH 6.4) both in presence and absence of thiosulphate. In controls ruminal fluid was replaced by distilled water. In all cases the initial concentration was 15 μg. HCN/ml. For analysis the units were cooled to 0°C., a few drops of 2 per cent NaOH carefully introduced, and the whole contents thoroughly shaken. The contents were then washed into the aeration tube and total HCN determined by the method of Gettler and Goldbaum. Results are shown in Table I.

TABLE I. LOSS OF HYDROCYANIC ACID FROM RUMEN FLUID *in vitro*

Composition of Reaction Mixture.	Total HCN (μg./ml.)	
	0 hr.	17 hr.
Distilled water and HCN	15	11
Ruminal fluid and HCN	15	1
Ruminal fluid, HCN and Thiosulphate (250 μg./ml.)	15	0

In the second series of experiments the HCN and rumen fluid together with sulphur compounds were incubated at 37° in burette tubes closed with tightly fitting bark stoppers. Ruminal fluid was previously filtered through glass wool to remove bulky fibrous material. The tubes were inverted frequently to mix the contents thoroughly, and samples were run out at intervals (the liquid in the tap being rejected) into 0.5 per cent NaOH and analysed for HCN. (The ruminal fluid itself contained 2-3 μg.H_2S/ml.) Results appear in Table II.

TABLE II. DECREASE IN HCN CONCENTRATION IN RUMINAL FLUID *in vitro*

Composition of Reaction Mixture.	HCN Concentration (μg./ml.)					
	0 hr.	½ hr.	1½ hr.	2 hr.	3 hr.	20 hr.
Controls : HCN alone	15				13	12
HCN, cystine (230 μg./ml.)	15			13	12	
HCN, ruminal fluid	15				10	5
HCN, cystine (230 μg./ml.) boiled ruminal fluid	15		13		12	
HCN, thiosulphate (230 μg./ml.) boiled ruminal fluid	15		12		12	
HCN, cystine (230 μg./ml.) ruminal fluid	15			5	0	
HCN, thiosulphate (230 μg./ml.) do.	15			6	1	
Control : HCN alone	3·0				3·0	3·0
HCN, ruminal fluid	3·0				2·6	0·0
HCN, cystine (42 μg./ml.), ruminal fluid	3·0	1·6	0·7			
HCN, thiosulphate (57 μg./ml.) do.	3·0	3·0	2·6	1·4		

The results show that in the absence of ruminal fluid there is a slight decrease in HCN concentration probably due to loss by diffusion. In the presence of ruminal fluid, however, there is a significant decrease in HCN, though this does not occur if the fluid is boiled before incubation. In the presence of cystine or thiosulphate ruminal fluid detoxicates HCN at a moderate rate, e.g., complete detoxication of 15

μg./ml. in three hours. Ruminal fluid alone slowly detoxicates HCN, complete detoxication of 15 μg./ml. taking 24-48 hours. This fact was verified on a number of occasions and is due to the initial concentration of H_2S and the slow release of sulphur donors from the food contained in the fluid.

In the previous paper it was shown that after dosing a sheep with KCN, most of the HCN had disappeared from the rumen within an hour. Even when allowance is made for possible differences between the *in vivo* and *in vitro* rates of reaction, the amount of HCN combined by the microflora in this period in the absence of added sulphur donors would be negligible. When sulphur or sulphur compounds are administered orally to stock as cyanide antidotes, however, it is possible that thiocyanate synthesis in the rumen may attain significant proportions. This would account in part for the observed beneficial results from oral administration of sulphur in cyanide poisoning, reported by Steyn (16).

Excretion of Free Hydrocyanic Acid

James (17) reported the recovery as free hydrocyanic acid in the urine and breath, of a large proportion of the sodium cyanide injected into human patients. Since it is possible that the extent of this method of removal varies from species to species an attempt was made to determine to what degree it occurs in sheep.

The apparatus used to trap the breath consisted of a leather mask which was fitted tightly around the muzzle of the sheep and from which the gases were continuously withdrawn by a mechanical pump at the rate of 30 litres per minute. Air entered the mask through an inlet after passing through a large flask, so that any expired gases forced back into the inlet would not escape before being swept back into the exhaust outlet. A measured fraction of the gases withdrawn from the mask was bubbled continuously through absorption tubes containing 7 per cent NaOH, and by estimating the HCN trapped in these the total HCN in the gases swept from the mask could be calculated. In control experiments a heated solution of ammonia was aerated and the air-ammonia mixture produced was directed into the mask. The amount of ammonia recovered from the absorption tubes (which in this case contained standard acid) indicated an ammonia content in the gases swept from the mask corresponding to over 99 per cent of the ammonia lost from the aerated solution. When similar controls were run with aerated cyanide solutions, however, the recovery was only 50-60 per cent.

In experiment 1 the Romney wether (body weight 70 kg.) was given 0.1 M-KCN through a rumen fistula. Breathing into the mask was commenced three minutes after dosing and continued for an hour with two short breaks. In experiment 2 in which a similar dose was given the same sheep, the animal was made to breath into the mask for alternate five-minute periods during an hour. The results, corrected for the low recoveries of HCN found in the controls, are given in Table III.

It is probable that not more than 10 per cent of the HCN administered to the sheep was exhaled in the breath. Determinations of HCN in the urine voided by sheep in the two or three hours following cyanide administration failed to reveal any excretion of free HCN by this means.

TABLE III. EXCRETION OF FREE HYDROCYANIC ACID BY SHEEP AND HUMANS

Expt. Subject.	Dose HCN. (mg.)	Percentage Recovery of HCN Administered		
		Breath.	Urine.	Saliva.
1 Romney wether	98	<19	—	—
2 Romney wether	98	5-6	—	—
3 Man	3·3	1·5	<0·2	0·0
4 Man	4·8	<3	<1·6	0·0

Since these results do not agree with those of James, an attempt was made to recover HCN from the breath, saliva, and urine, of human subjects to whom a small dose of 0.1 M-KCN had been administered orally. In experiment 3 the subject (body weight 70 kg.) exhaled through glass tubing into 7 per cent KOH for 30 min. after cyanide administration. Urine was collected for the hour following the dose. In experiment 4 the subject (body weight 65 kg.) exhaled into caustic soda solution for alternate 5-minute intervals for one hour. Urine was collected for the two hours following the dose. Results appear in Table III.

Even though experimental error was large the results undoubtedly indicate that both in sheep and humans the amount of free HCN excreted in breath, urine, and saliva, after cyanide administration is negligible. The discrepancy between our results and those of James probably arises from the difference in the methods used for HCN analysis. While the method of Gettler and Goldbaum used by us is specific for HCN, the tests used by James are considered by Gettler and Bain (18) and by Van der Walt (19) to give positive results with several other compounds. Our results are also in better agreement with the observed excretion of a large proportion of administered cyanide as thiocyanate. As a further rough proof that HCN is not exhaled, the breath of the fistulated sheep and those dosed in the toxicity trials (Part III) was smelt. Although a human can detect 1-5 μg.HCN by smell, no HCN was ever detected in the breath. There is one possible exception to this. The gaseous cavity, often occurring above the fluids in the rumen, does contain HCN and some loss should therefore occur when the animal belches but belching is so infrequent and small that the loss by this method is insignificant.

THIOCYANATE IN URINE AFTER CYANIDE ADMINISTRATION

In order to determine whether cyanide could be recovered quantitatively as thiocyanate in the urine, the fistulated sheep was placed in a metabolism crate for several days for collection of urine. Urine was collected every 24 hours, toluene added, and stored in a refrigerator until analysed. The recovery of thiocyanate added to urine and estimated by the method of Baumann, Springson and Metzger, was 87 per cent. All thiocyanate values recorded below have been corrected for the lack of complete recovery in estimation.

It was found from observations on two sheep that the normal thiocyanate excretion on a diet of lucerne and timothy hays was of the order of 1 mg.HCNS per day, or 0.02 mg.HCNS/kg./day. The daily excretion of thiocyanate following cyanide administration is given in Table IV.

TABLE IV.

		mg.	mg.
HCN dosed		568	120
HCNS equivalent		1,240	262
HCNS recovered	1st day	244	22
	2nd day	378	32
	3rd day	81	13
	4th day	—	—
	5th day	—	—
	6th day	5	—
Percentage Recovery		57	26

Two significant observations arise from these figures. Firstly, even allowing some increase for the days on which urine was not collected, the percentage recovery is far from 100 per cent, and is probably of the order of 60-70 per cent for large doses. It is possible that the urine is not the only medium of excretion and that some may be excreted in the fæces and as suint. The failure to obtain complete recovery in the urine may mean either that thiocyanate formation is not the only mode of detoxication or that the urine is not the only medium of excretion. The second observation is that excretion is very slow, occurring over a period of several days, and reaching a maximum on the second day.

CYANIDE AND THIOCYANATE IN THE BLOOD AFTER CYANIDE ADMINISTRATION

Since it has been shown that HCN is rapidly absorbed from the rumen and that it is probable that thiocyanate formation is largely responsible for the detoxication of HCN, it would be expected that blood concentrations of cyanide and thiocyanate after cyanide administration would give some indication of the rates of these processes.

The fistulated sheep was given 0.1 M-KCN (corresponding to 91 mg. HCN) via the rumen fistula, and blood samples were taken after ½, 1, 2, 5, 24, hours. At each sampling about 10 ml. of blood were run into a cold oxalated bottle which was immediately stoppered, shaken, and placed in a freezing mixture. A further 30 ml. of blood were run into a centrifuge tube and allowed to stand at 0° until clotting had occurred.

TABLE V. BLOOD CYANIDE AND THIOCYANATE CONCENTRATIONS AFTER ADMINISTRATION OF KCN AND KCNS

Sheep.	Body weight. (kg.)	Dose. (mg.)	Analysis.	Concentration. (μg./ml.)							
				(Min.)			(Hr.)				
				7	15	30	1	2	5	18½	24
Romney Wether	70	HCN 91	Blood HCN		0.5	0.35	0.2	0.2	0.1		
			Increase in serum HCNS		0.2	1.2	1.8	2.2	1.9		1.5
Romney Wether	70	HCNS 68	Increase in serum HCNS	1.4	2.4			1.8		2.0	
Corrie-dale Hogget	32	HCNS 34	Increase in serum HCNS		2.5		2.1	1.8			

The oxalated blood samples were analysed for cyanide by the method of Gettler and Goldbaum, and serum thiocyanate of the clotted samples was determined by the method of Aldridge. The results obtained together with serum thiocyanate values after injection of 0.1 M-KCNS are given in Table V.

Sheep serum thiocyanate ranged from 5.1 - 9.2 μg. per ml. with a mean value of 7.2 μg. per ml.

In an experiment (Experiment No. 2, Part III) in which the Romney wether was given repeated doses of 0.1 M-KCN via rumen fistula, the following values of serum thiocyanate were recorded (Table VI).

TABLE VI. SERUM THIOCYANATE DURING CONTINUED DOSING OF KCN

Time from Commencement of Dosing. (hr.)	Dose administered up to time of Sampling. (mg. HCN)	Serum HCNS. (μg./ml.)
0		7·0
9½	714	28·0
14¾ (immediately after last dose)	1070	35·3
33¾ (19 hr. after last dose)	1070	16·5
50¾ (36 hr. after last dose)	1070	6·4

It may be seen from Table V that in cases where a single dose of cyanide was administered, the blood HCN concentration reached a maximum of about 0.5 μg./ml., within 15 minutes, and then steadily decreased over the next few hours. In sheep given fatal doses of cyanide the average post mortem concentration of HCN was 1.0 μg./ml. in the blood, and 0.5, 0.6, 2.0 μg./g. of moist tissue in kidney, muscle, and liver, respectively. In the case of each tissue the range of concentrations found was small. These results suggest that normally cyanide is rapidly detoxicated in sheep, and that the critical concentration in the body which produces death is comparatively low.

On the other hand in the sheep subjected to continued dosing relatively high serum thiocyanate concentrations were obtained, and about 48 hours elapsed before the level fell to normal. Also after single doses of KCN or KCNS the time required for the serum thiocyanate level to return to normal was over 24 hours. This indicates that the thiocyanate remains in the system for some time, being excreted over a period of 1-2 days, a result confirmed by urine analyses.

ENZYMIC SYNTHESIS OF THIOCYANATE FROM CYSTINE AND HCN

It is apparent that detoxication in the sheep must be due primarily to a rapid synthesis of thiocyanate. Lang's conclusion that thiosulphate or colloidal sulphur is the sulphur donating compound seems difficult to reconcile with the fact that these materials are not known to be available in any significant quantity in the animal. It therefore seemed desirable to investigate the utilization of sulphur compounds known to be available in the animal, and to extend these studies to the sheep.

Extracts of sheep liver were prepared by extracting 100 g. of fresh liver with 200 ml. of phosphate buffer, pH 7.6, in a Waring blendor. On centrifuging, rather less than 200 ml. of extract were obtained. Normal saline extracts were of about the same activity as phosphate extract. Extracts were relatively stable but slowly lost their activity

on standing, even when kept at 0°C. In each experiment 10 ml. of extract were used in a total volume of 20 ml. final concentrations of cyanide were kept in the range 2.5 - 20 μg.HCN/ml. to approximate to body concentrations as closely as analytical methods permitted. Phosphate was not essential to thiocyanate synthesis, the reaction proceeding just as rapidly in unbuffered solutions or with borate buffers as with phosphate buffer. The reaction mixture was usually incubated at 37°C. in burette tubes closed with bark stoppers so that samples could be run out at intervals. Mixing was effected by inverting at intervals. In the experiments to determine accurately the reaction rate using H_2S, the mixture was simply placed in a stoppered tube which was maintained at 37°C., samples being pipetted out at intervals. Toluene was found to be without effect on the enzymic reaction and 2 per cent was added to retard bacterial action.

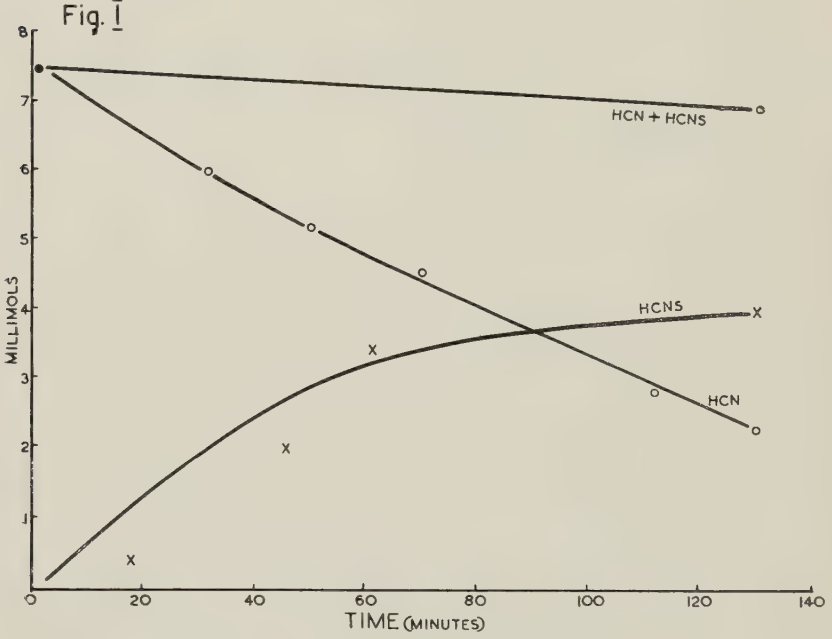
Synthesis of thiocyanate by liver extracts in presence of cyanide and cystine was first investigated. Using cystine, dissolved in sodium carbonate solution to give a concentration of 0.0025 M, and with an initial HCN concentration of 10 μg./ml., the time for 50 per cent decrease in HCN concentration was about 45 min. The reaction was usually carried out aerobically but it proceeded at essentially the same rate when carried out under nitrogen. By shaking the crude phosphate extract with an equal volume of chloroform for 20 minutes part of the protein material was precipitated out. On centrifuging a clear supernatant liquid was obtained the volume of which was about one-third that of the extract used. If the activity of the extract is expressed as $\frac{10^4}{T \times M}$ where T is the time in minutes for 50 per cent decrease in HCN concentration and M is the number of mgs. of Kjeldahl nitrogen, the activities of the crude phosphate and chloroform treated extracts were 2.5 and 4.0 respectively. Activities of successive preparations from livers of different animals showed only small deviations from these figures. With an initial HCN concentration of 20 μg./ml. time to 50 per cent decrease in HCN concentration was about 160 min., using the chloroform treated extract. Kidney extracts had somewhat lower activity than liver extract, and muscle and blood possessed negligible activity.

The effect of pH on the synthesis of thiocyanate by liver extract in presence of cystine and HCN was measured. The optimum pH was 8.1 and at pH 7.1 and 9.2 the activity was 80 per cent of optimal. The curve is similar to that obtained by Lang for the synthesis using thiosulphate as the sulphur donor, for which he obtained an optimum pH of 8.3.

When extracts were boiled the solid material formed made it necessary to filter or centrifuge before the treated material could be tested for activity. The clear liquid so obtained by boiling active extracts was always inactive. These facts make it probable that the active material is proteinaceous in nature, a fact confirmed by the effect of trypsin on the extract. After addition of trypsin to active extract to give a 1 per cent suspension and incubation at 37°C., the activity was reduced 18 per cent after 1 hour and 92 per cent after 25 hours.

In order to show that loss of HCN was due to thiocyanate synthesis, reactions were carried out in which simultaneous analyses for HCN and HCNS were made. The thiocyanate formed accounted for 60-90 per

cent of the cyanide lost, the magnitude of the difference depending on the methods of analysis used. A typical result is shown in Fig. 1. The HCN concentration in control experiments in which cystine was omitted, decreased at about the same rate as that at which the sum of cyanide and thiocyanate decreases in Fig. 1. A small amount of thiocyanate synthesis was observed in controls in which cystine was omitted. Tests showed that α-amino-β-thiocyanopropionic acid did not give a reaction in the thiocyanate analysis.



The only mechanism suggested for the direct reaction of cystine and HCN *in vivo* is that put forward by Voegtlin, Johnson, and Dyer (5). They considered the reaction to be

$$RSSR + H_2O + NaCN \longrightarrow 2\,RSH + NaCNO.$$

However they did not demonstrate cyanate formation, and such a reaction is not compatible with the observed formation of thiocyanate. At higher concentrations cystine and HCN react according to the equation $RSSR + HCN \rightleftharpoons RSH + RSCN$.

Cysteine formation from cystine and HCN incubated in the presence of purified liver extract, was proved by the development of a positive nitroprusside reaction. Liver extract itself gives a positive reaction but when it was purified by precipitation with alcohol and subsequent washing with alcohol or acetone, the liver itself, when incubated alone or with HCN and cystine separately, gives no nitroprusside reaction. HCN and cystine without liver extract likewise gave no reaction. Hence liver extract brings about the reduction of cystine to cysteine by HCN. In order to show whether the formation of cysteine was related to decrease in HCN concentration, simultaneous analysis for HCN and cysteine were carried out. To give satisfactory cysteine analyses the initial concentrations of HCN and cystine had to be increased to 0.0015 M. Results are given in Table VII.

TABLE VII. CYSTEINE PRODUCTION FROM CYSTINE AND HCN IN PRESENCE OF LIVER EXTRACT (pH 7·6) INCUBATED AEROBICALLY AT 37°C.

Time of Reaction. (hr.)	Cysteine formed. (Moles per 100 ml. × 10^5)	HCN decrease. (Moles per 100 ml. × 10^5)
½	2·8	3·9
1	5·3	8·8
2	11·8	13·8
4	21·5	16·0

These results indicate that the first step in the reaction is probably

$$RSSR + HCN \longrightarrow RSH + RSCN$$

followed by enzymic decomposition of the alkyl thiocyanate to form inorganic thiocyanate. Lang (20) demonstrated the slow formation of inorganic thiocyanate from esters of thiocyanate and isothiocyanic acids in presence of cat liver. The formation from the alkyl ester was much more rapid than from the methyl ester. Hence it is probable that the liver could rapidly decompose α-amino-β-thiocyanopropionic acid formed as indicated above. The slow formation of thiocyanate and α-amino-β-thiocyanopropionic acid in small amounts from alkaline solutions of cystine and KCN has been reported by Meyer and Bodansky (3). Cysteine, at the same molar concentration as the cystine, gave somewhat variable results but the rate of thiocyanate synthesis approximated that obtained with cystine. Reaction in this case might be due to preliminary oxidation to cystine, a reaction which has been shown to be catalysed by liver cytochrome oxidase. However, preliminary degration of cysteine and cystine might also play some part. Smythe (21) reports that the desulphurase of the liver, producing H_2S from cystine and cysteine, is strongly inhibited by As_2O_3. Addition of As_2O_3 to give a concentration of 0.01 M. caused no inhibition of thiocyanate formation from cystine and cysteine, indicating that preliminary production of H_2S played no significant part in the reaction. Hydrazine sulphate at a concentration of 0.01 M. caused some inhibition (10-40 per cent) of thiocyanate formation from cysteine and cystine, but in this case the effect was probably due to the reducing action rather than to inhibition of desulphurase. The fact that desulphurase appeared to take no part in the reaction is in accord with Smythe's observation that the enzyme is inhibited by 0.007 M-KCN, as indicated by H_2S and NH_3 production. Preliminary incubation of the cystine or cysteine with the liver extract for two hours at 37°C. before addition of cyanide caused a considerable increase (2-4 times) in the rate of thiocyanate production. Addition of As_2O_3 during the incubation period reduced the increase by about 30 per cent.

Incubation of HCN with S-benzylcysteine in presence of liver extract gave no synthesis of thiocyanate. Methionine caused removal of HCN at about half the rate obtained with an equimolar amount of cystine.

Since heavy metal cations are known to catalyse a number of reactions of cystine and cysteine, the effect of various cations on the thiocyanate synthesis from cystine was investigated. The sulphate of Ca, Fe, Mg, Zn, Mn, and Cu, respectively, were added to give concentrations of 0.01 M. Only the last three produced any significant effect,

giving 60-90 per cent inhibition. Their action was possibly due to insolubility of the cyanides. Similarly compounds known to inhibit reactions catalysed by heavy metals produced no inhibition of thiocyanate synthesis. 0.001 M-8-hydroxyquinoline, 0.01 M-sodium diethyldithiocarbamate, 0.01 M-thiourea and, 0.01 M-sodium pyrophosphate, were all without effect.

THIOCYANATE SYNTHESIS FROM OTHER SULPHUR COMPOUNDS

HCN was found to react rapidly and quantitatively with both sodium thiosulphate and sodium sulphide in presence of liver extract (pH 7.6) at 37°C. When the initial concentration of the reactants was 10^{-4} to 10_{M-5} 50 per cent synthesis of thiocyanate was accomplished in a few minutes. The influence of initial concentration of the reactants on the rate of aerobic thiocyanate synthesis by liver extract using sodium sulphide is shown in Fig. 2.

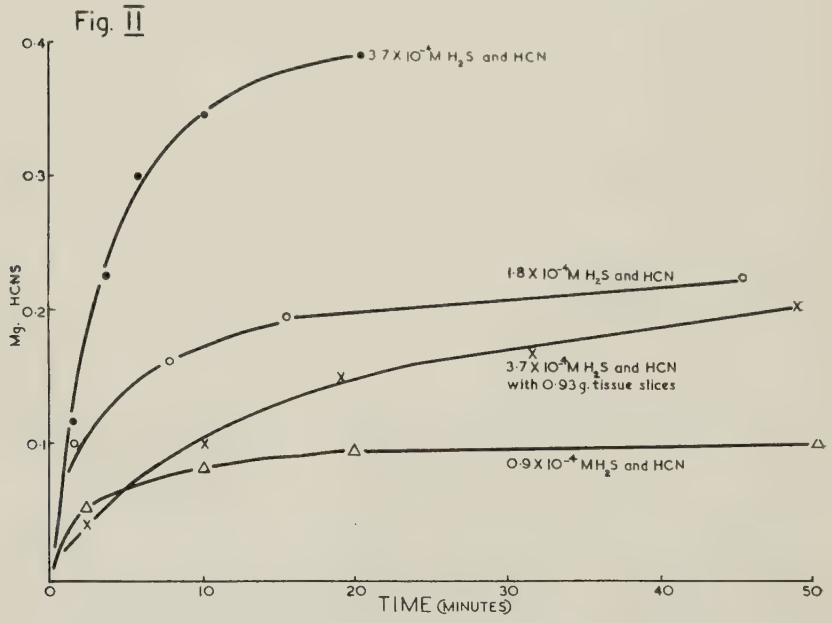


Fig. 2 also shows the production of thiocyanate from sodium sulphide by a suspension of liver slices in Ringers solution at 37°C. Considering that only 0.93 g. of liver slices were used, whereas extract from 5 g. of moist liver was used for the other results, the reaction rate is relatively high, especially since under these conditions it largely depends on the rate of diffusion of substances through the slices.

According to Lang (6) sulphides can be utilized for enzymic thiocyanate synthesis only to the extent that oxidation to elementary sulphur occurs. To investigate whether oxidation to elementary sulphur is necessary, the reaction with sodium sulphide was carried out under nitrogen. No change in the rate of reaction could be detected by making the reaction anaerobic. Even when the reaction was carried out under nitrogen in presence of 0.01 M-hydrazine sulphate the rate was unaltered. Hence it is unlikely that preliminary oxidation to sulphur occurs. If it does it can proceed anaerobically. To verify this conclusion

the rates of thiocyanate synthesis in presence of sodium sulphide and of sodium polysulphide were compared. If formation of elementary sulphur is a prerequisite for thiocyanate formation it would be expected that sodium polysulphide would give a more rapid reaction than an equimolar amount of sodium sulphide. No change in reaction rate could be detected when polysulphide was substituted for sulphide.

The activity of liver extract for thiocyanate synthesis from sulphide or thiosulphate is destroyed by boiling or by incubating with a 2 per cent trypsin suspension. The active material must therefore be proteinaceous in both cases.

Sodium nitrite is frequently administered as an antidote for cyanide poisoning, acting by forming methaemoglobin which combines with cyanide to form cyanomethaemoglobin. To determine whether in addition to this effect sodium nitrite influences thiocyanate formation, the enzymic synthesis of thiocyanate utilizing thiosulphate and cystine respectively was carried out in presence of sodium nitrite. No change in the rate of the reaction could be detected so that it may be concluded that nitrite plays no part in the thiocyanate synthesis.

Effect of Glucose

Since the antidotal action of glucose is generally attributed to cyanhydrin formation it is to be expected that the concentration of HCN in serum and in liver extract would decrease in presence of glucose. When HCN (initial concentration 10 μg./ml.) and glucose (250 μg./ml.) were incubated at 37°c. in presence of serum or liver extract a slow decrease in HCN content was observed. For serum the loss was 30 per cent in four hours, and for extract 50 per cent in four hours. Loss of HCN without addition of glucose was slower, amounting to about 70 per cent in 20 hours for serum. A small part of the loss of HCN was due to thiocyanate formation, but for the most part cyanhydrin formation was probably responsible. However it will be seen that an *in vivo* reaction rate similar to that obtained in these experiments would render the process quite ineffective as a detoxication mechanism. Similar results were obtained by Kohn-Abrest and Lupu (23). The loss of HCN from deproteinated blood at 37°c. in presence of an amount of glucose corresponding to that in normal blood, was 68 per cent in two days. These results give no support to the conception of cyanhydrin formation as an important *in vivo* process. Voegtlin, Johnson, and Dyer (5) and other authors have found glucose ineffective as an antidote to cyanide poisoning, and Chauchard *et al.* (23) found that reducing sugars did not prevent the decrease in chronaxia produced in rats by injection of cyanide. It should be further noted that in cases where reducing sugars, aldehydes, or ketones, have been found to inhibit *in vitro* the physiological effects of KCN, the concentrations of HCN and of the carbonyl compounds were usually much higher than those attained *in vivo*. There seems to be considerable doubt, therefore, whether cyanhydrin formation does occur sufficiently rapidly to be of value to the organism. The oral administration of glucose produces little change in the blood glucose level, and hence is unlikely to influence the formation of cyanhydrin unless this occurs in the rumen, which has been shown improbable in Part I. The results of those who found glucose an effec-

tive antidote for HCN may possibly be explained by the small numbers of animals often used, or by secondary effects on carbohydrate metabolism which is profoundly affected by cyanide (30).

Production and Absorption of H_2S in the Rumen

H_2S is produced from ingested food in the normal functioning of the rumen. Analyses of ruminal fluid for H_2S showed normal values of the order 1 - 4 μg./ml. when the sheep was eating lucerne and timothy hay, 3 - 5 μg./ml. when eating green pasture, and greater than 5 μg./ml. when eating cruciferous foods.

Since H_2S is a volatile acid one would expect it to be absorbed through the ruminal wall in the same manner as for HCN and the volatile fatty acids. To test this the fistulated sheep was dosed, per fistula, with 100 mg.H_2S in the form of sodium sulphide solution to which an equivalent amount of acid had been added. Concentrations in the rumen at intervals are given in Table VIII. The method of estimation determines free H_2S plus sulphide. The pH of the rumen was 7.4.

Table VIII. Sulphide + H_2S Concentrations in Rumen after Dosing with 100 mg. H_2S

Time.	Concentration (as μg.H_2S/ml.)
Before Dosing	2·7
10 minutes after dosing	16
20 ,, ,,	10
30 ,, ,,	11
50 ,, ,,	6·5
80 ,, ,,	3·8

These results show that H_2S does disappear from the rumen, probably by absorption, at a rate which is considerable but notably less than that for HCN. This is to be expected for whereas the K_a for HCN is 7.2×10^{-10}, the value for H_2S is 9.1×10^{-8}. Hence within the normal range of pH values of the rumen significant amounts of H_2S occur as sulphide ions which are much less readily absorbed through the ruminal wall. At the pH obtaining in the above experiment, namely 7.4, more than half of the sulphide is present in the ionic form. After making due allowance for this it can be said that the absorption of free H_2S is rapid. At pH's lower than about 6.5 the rate of absorption of H_2S would be considerably greater than in the experiment quoted.

Discussion

The rapid synthesis of thiocyanate by tissue extracts indicates that thiocyanate formation is probably the main means of detoxication of cyanide in sheep. This is supported by a marked increase in blood thiocyanate following cyanide ingestion and by recoveries in the urine of over 50 per cent of administered cyanide as thiocyanate. At the same time the fact that 100 per cent recovery was not obtained casts some doubt on thiocyanate formation being the only method of detoxication. Detoxication within the rumen has been shown to be possible though slow, and removal of free HCN by exhalation has been shown to be of a low order. If doubt exists as to all the means of detoxication at least it is certain that thiocyanate formation is the most rapid and most important. The source of sulphur for this process in the animal body is far less certain however. Although cystine is known to be present in

animal tissues both free and in peptide form, the observed rate of reaction with HCN *in vitro* could perhaps be considered to indicate that the *in vivo* reaction does not utilize the intact molecule of cystine or cysteine. The antidotal effect of cystine, cysteine, and glutathione for cyanide poisoning reported by Voegtlin, Johnson, and Dyer (6), may involve preliminary degradation of the injected compounds with formation of H_2S and thiosulphate which are immediately utilized for the thiocyanate synthesis. That such a rapid degradation of administered cystine occurs normally is suggested by the work of Debrenci (25) and of Hier (26). Debrenci showed that in dogs with Eck fistula cystine exerted a hyperglycemic action which did not occur in intact animals, and concluded that normally this substance is rapidly broken down in the liver. Hier found that in the dog the plasma cystine level was not raised by ingestion of 0.56 g. cystine per kg. body weight, indicating that the organism can rapidly remove the cystine from the plasma either by excretion, conversion, or storage.

Thiosulphate does not occur in appreciable amounts in the animal body although Garabidian and Fromageot (26) have shown that it is probably formed as an intermediate in the oxidation of H_2S. However, H_2S is normally formed in the rumen and intestine and is a normal constituent of the rumen gases. Dougherty (27) found as much as 0.03 per cent by volume of H_2S present in the rumen gas of cattle and 0.309 ml. per 100 ml. of rumen ingesta ($= 4\ \mu g./ml.$). In the fistulated sheep values of 1 - 5 $\mu g.H_2S/ml.$ have been observed and appear to be normal. Further, this H_2S is absorbed at a considerable rate depending mainly on the pH of the rumen.

Rapid oxidation of H_2S in the organism under normal conditions is indicated by the finding of Tichonravov (28) that intravenously injected H_2S rapidly disappears from the blood of the dog. This presents a considerable difficulty to the view that H_2S is utilized for thiocyanate formation. However, in presence of cyanide, H_2S is possibly utilized for thiocyanate formation in preference to oxidation. In this connection it is perhaps significant that cyanide decreases the rate of oxidation of H_2S in the blood, plasma, liver, spleen and skin of the rabbit (29). In the fasting or postabsorptive state H_2S for detoxication of cyanide cannot be derived from the rumen or intestine but might be obtained by degradation of cystine mobilized to the liver. The rapid rate at which cystine can be metabolized or stored when ingested suggests that such an emergency mobilization may be possible and Hardovsky (30) has noted an increase in free glutathione in the muscle of rabbits poisoned by HCN. Such a dependence on endogenous H_2S would be in agreement with the lowering of the lethal dose of HCN by prolonged fasting as described in Part III of this series. Administration of sulphur compounds greatly increases the amount of HCN tolerated so that the limiting factor for HCN tolerance could be, under certain conditions, the rate at which H_2S can be supplied by the organism, rather than the capacity of the enzyme system. Forst (4) has shown that protective doses of thiosulphate or colloidal sulphur are limited in their effect, the limit of effectiveness lying near 3-4 times the lethal dose. This level would represent the maximum capacity of the enzyme system.

Fig. 2 indicates that at the concentrations of HCN occurring *in vivo* 50 per cent removal of HCN occurs in about 2 min. in the

presence of H_2S. With an HCN concentration of 2.4 μg./ml. (being the 0.9×10^{-4}M concentration of Fig. 2) this would give a rate of combination of about 150 mg. of HCN per hour, or 2.1 mg./kg. assuming that the weight of the liver and kidneys in a 70 kg. sheep is about a kilogram and that an equivalent amount of H_2S is available. However, it is probable that the intact organ functioning normally would carry out the reaction at a considerably greater rate than that of the extracts *in vitro*. Furthermore, no steps were taken to make the extraction of the enzyme from the liver exhaustive, so that the estimated rate is probably 30-50 per cent too low on this account alone. An *in vivo* reaction rate of about twice that obtained by the extracts *in vitro* would give a rate of thiocyanate formation equivalent to the maximum rate at which HCN can be tolerated, as demonstrated in Part III.

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STUDIES ON A FUNGUS (*Septoria pisi* Westend.) CAUSING A FOLIAGE DISEASE OF PEAS (*Pisum sativum* L.)

By I. A. M. CRUICKSHANK,* Canterbury Agricultural College, Lincoln

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Summary

Septoria-blotch (*Septoria pisi* Westend.) is recorded on both field and garden peas (*Pisum sativum* L.) throughout New Zealand. Symptoms consist of light brown amphigenous broad irregular lesions on both stems and leaves. Small black pycnidia are scattered evenly over the lesions on older leaves.

Pathogenicity has been established.

The chief cultural characters are the campanulate form of the colonies, their olive green colour, their very slow growth, and tardy sporulation. Pycnidia are 79.0-237.0μ diameter; the wall is of loosely interwoven hyphae, and the ostiole ill-defined. Spores 24.5-59.0 $\times$ 3.0-3.5μ are cylindrical, straight or irregularly curved, and 1-3 septate.

The classification of *S. pisi* in the genus *Septoria* is discussed.

INTRODUCTION

A FUNGUS disease not previously recorded in New Zealand was observed on crops of field peas in Canterbury (Lincoln) during disease survey work in the 1946-47 season. This disease has also been found on crops of garden peas in Rangiora, Wanganui and Auckland. Using field material at the outset, an investigation was initiated by the author, the significant features of which are outlined in this paper.

SYMPTOMS

The first noticeable symptoms are small yellowish areas, which develop into light brown necrotic blotches. The latter are broad, irregular, elongated and limited by the veins of the leaves (Fig. 1). Minute light brown sub-epidermal pycnidia, which darken with age appear scattered over the diseased areas (Fig. 2). The disease spreads over leaves, petioles, stipules and stems. Severe infection of young plants results in shrivelling of leaves and shrinking and girdling of the stems, followed by wilting and death. On older plants the symptoms are similar but the results not as severe.

ISOLATION AND PATHOGENICITY

The fungus was isolated from spore horns exuded from pycnidia after infected portions of dry unsterilized leaves had been held in petri dishes lined with moist filter paper for several hours. This was done by removing small segments of the spore horns with a sterilized needle and plating directly upon standard potato dextrose agar. Single spore cultures were prepared from spore suspensions.

Pots of steam-disinfected soil were sown with surface-disinfected seed (Mercuric chloride 1-1000 for 3 min.) of Partridge and Austrian winter varieties of field peas. After two months' growth in the glasshouse, plants were inoculated as follows:—Aerial portions of the plants were enclosed in 3 in. diameter glass cylinders and held at 20-21°c. in

* Now at Plant Diseases Division, Department of Scientific and Industrial Research, Christchurch.

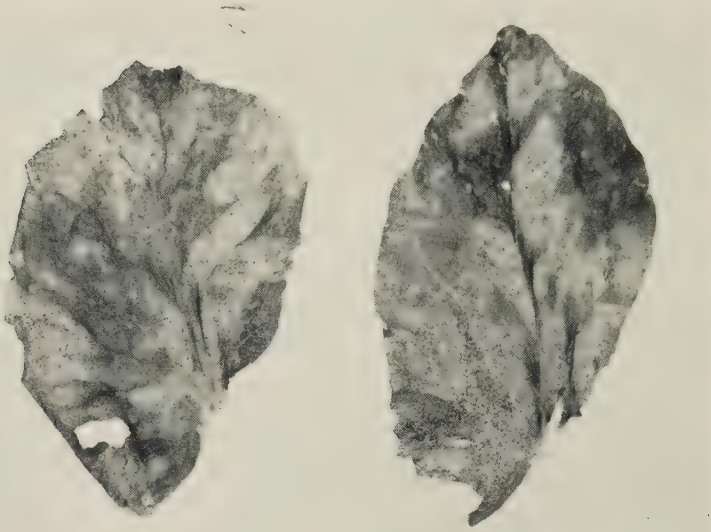

FIG. 1.—Pea leaves showing typical field symptoms of septoria-blotch, ×1.

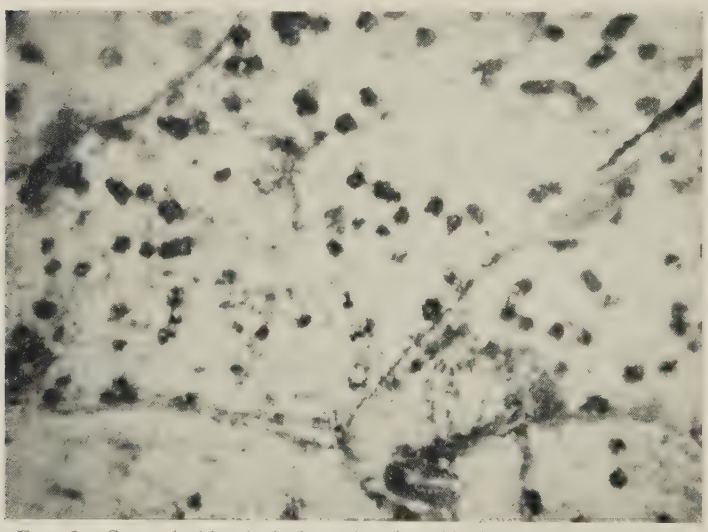

FIG. 2.—Septoria-blotch lesion showing black pycnidia scattered over surface of leaf (enlarged).

a constant temperature room. The ends of the cylinders were stoppered with cotton wool, so that the upper cotton wool bung could be removed for spraying purposes. Twenty-four hours prior to inoculation the foliage was moistened with sterile water, inoculation was then done by spraying the plants with a spore suspension in sterile water prepared from cultures of the fungus grown on standard potato dextrose agar. Three to four days after inoculation, the glass cylinders were removed, and plants placed in a shady position in a glasshouse. Check plants were sprayed with sterile water.

Symptoms of disease became visible after 12-14 days.

CULTURE STUDIES

Macroscopic characters:

A range of agar media,* prepared according to the formulæ of Riker and Riker (1936), was employed to determine the colony characteristics of *S. pisi*. Four to five days after inoculation single spore isolants were visible as minute flesh-coloured colonies, and as growth continued they developed both in area and in height above the surface of the media. Growth on all media was very slow. One colony on standard potato dextrose agar, held at 22°-23°C. for two months, grew to a diameter of 35 mm. (Fig. 3).

FIG. 3.—Culture of *S. pisi* held at 22-23°C. for two months (approx. ×1).

Maximum growth was obtained on pea extract agar. Hydrogen ion concentration had no apparent effect on growth within the range pH 5.0-6.0, but outside this range, growth was very restricted. The optimum temperature for growth was 20°-22°C. but growth was obtained over the range 12°-25°C.

On vegetable extract agar, mature colonies were campanulate while on Czapek's sucrose agar growth was chiefly beneath the surface of the medium. Most colonies were olive green in colour but colonies on bean extract were dark brown and those on potato plugs were white. Old colonies exhibited distinct concentric zonation and clear droplets appeared over the surface of the colonies.

* Potato dextrose agar, Carrot extract agar, Pea extract agar, Cornmeal agar, Malt extract agar, Oatmeal agar, Bean extract agar, Czapek's sucrose agar, Potato plug, Bacto Prune agar, Bacto Nutrient agar.

Microscopic characters: —

The colonies consisted of masses of smooth and irregularly verrucose, very thick-walled dumb-bell shaped cells (Fig. 4c) together with narrow thin walled aerial hyphae, 2.0-2.5μ diameter, and thick-walled submerged hyphae, 3.5-4.0μ diameter. In both latter types a septum was observed at the junction of hyphal branches. Oil droplets, which stained red with Sudan III, were observed within both thick-walled hyphal types.

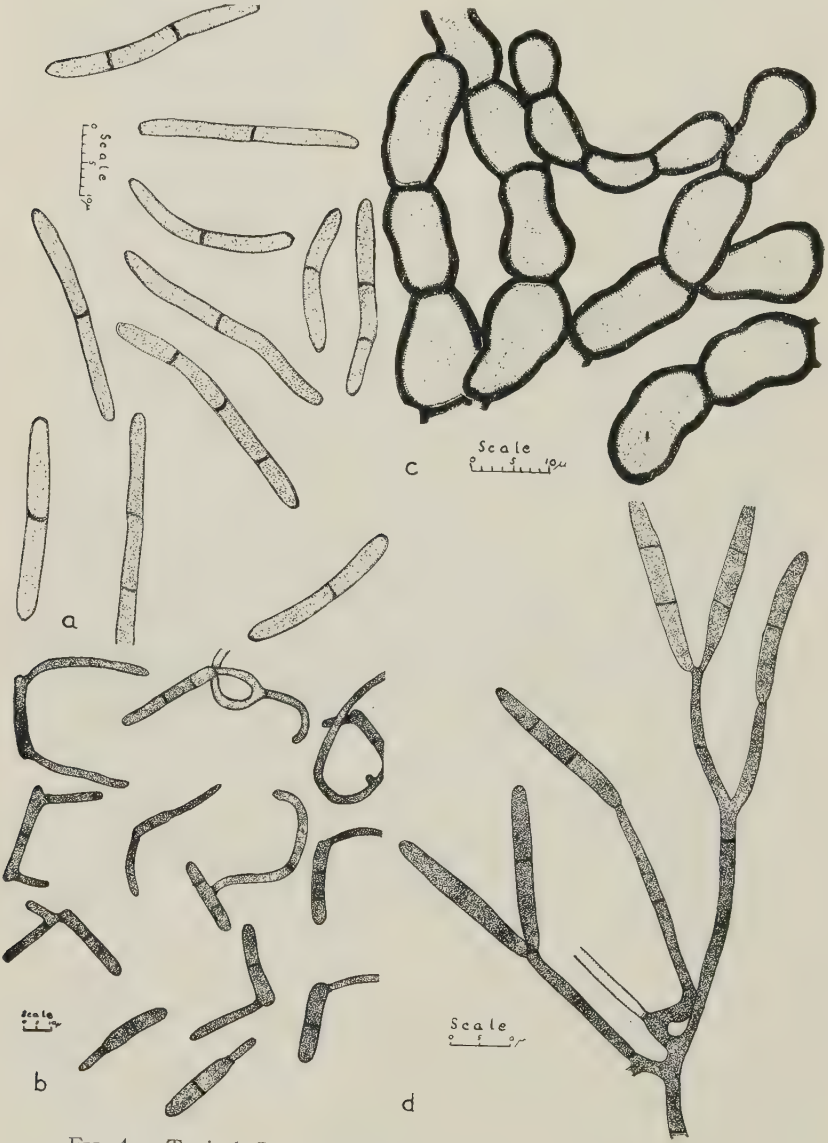

FIG. 4a.—Typical *S. pisi* spores.
b.—Germined *S. pisi* spores.
c.—Thick walled dumb-bell shaped cells from culture.
d.—Production of *S. pisi* spores in culture. Original.

Sporulation was very tardy on the wide range of media employed and in spite of efforts to induce sporulation by physiological shock and the use of media of high cellulosic content* most cultures failed to fruit. In the few instances that sporulation did occur the spores were produced in spore horns, elevated above the colony surface. The spores were cut off in basipetal succession from long conidiophores (Fig. 4d). Both in dimensions and septation, these spores were identical with those produced on diseased host tissue.

MORPHOLOGY STUDIES

Diseased leaf tissue was fixed in Bouin's fluid under reduced pressure, and microtome sections were prepared using n-butyl alcohol (Zirkle, 1930). Thionin and orange G (Stoughton, 1930) were used to stain the pathogen in the host tissues.

Typical mature spores were obtained from spore horns when diseased leaves were held in petri dishes lined with moist filter paper. Temporary preparations were made using lacto-phenol red as mountant. The spore staining technique of MacMillan and Plunkett (1942) was also employed. This consisted essentially in fixing spores to a slide by gentle heat, followed successively by staining with a warmed 10 per cent aqueous solution of sodium hydroxide and a saturated aqueous solution of picric acid.

Spore germination in sterile water and on standard potato dextrose agar was studied in van Tieghen cells. Examinations for spore germination and hyphal penetration were made with pieces of epidermis obtained from inoculated leaf material incubated at 22°-23°C. for a period of four to five days in humidity chambers.

Vegetative hyphae:

Throughout diseased leaf tissues there developed intra- and intercellular, hyaline, regularly septate, thin-walled hyphae, 1.5-2.0μ diameter.

Pycnidia:

Pycnidia (Fig. 5) occurred beneath both upper and lower epidermis of the leaf and in both pallisade and mesophyll tissues—frequently

FIG. 5.—Typical mature pycnidium of *S. pisi* in leaf tissue. Original.

* Steamed pea stems and sterilized dry pea and wheat straw.

beneath the stomata. The walls of the pycnidia were thin, and composed of loosely interwoven hyphae, 3.0-3.5μ diameter. Conidiophores bearing spores singly or in pairs, arose from the pycnidial wall. Ostioles were wide and ill-defined.

Spores:

Light brown spore horns or cirri exuded from the pycnidia. They were cylindrical, thread-like, straight or slightly curved, 1.0-1.5 mm. long, and approximately circular in cross section. Spores were held together by mucilage which rapidly disintegrated in water, thus releasing the spores.

Typical spores were cylindrical, generally straight, hyaline, 1-3 septate, 24.5-59.0μ $\times$ 3.0-3.5μ (av. 33.0 $\times$ 3.2μ) (Fig. 4a). Guttulae frequently described in the genus *Septoria* were not observed. Induction of plasmolysis in mature spores followed by examination under a dark field revealed an integument which enclosed 2-4 separate protoplasts, each of which was limited by a plasma membrane. This demonstration confirmed the view of MacMillan and Plunkett (1942) that no true septation occurred in the spores of some species of *Septoria* but that apparent septation* was caused by the abutting plasma membranes of contiguous cells within the integument.

Spore germination was initiated by slight swelling in spore diameter but no increase in length. At germination the integument was ruptured by the germ tubes which were developed from both median and terminal protoplasts of the spores (Fig. 4b). Germ tubes produced in water and on agar smears were only slightly less in diameter than the parent spores (3.0-3.5μ diameter). Germ tubes from spores germinating on host tissue were 1.5-2.0μ diameter.

Direct penetration of epidermal cells was effected by recurved hyphal tips branching from primary germ tubes. No stomatal penetration was observed.

DISCUSSION

S. pisi was first described by Westendorp in 1861. Subsequently there has been recorded in the literature a number of investigations describing features of a foliage disease attributed to *S. pisi*, notably Potebnia (1910) in Russia, Kovachevsky (1938) in Bulgaria, Melhus (1913), Linford (1929), Weimer (1936), Zaumeyer (1939, 1942) and Walker and Hare (1942) in the United States of America.

The position of *S. pisi* in the form genus *Septoria* offers some doubt. MacMillan and Plunkett (1942) considered that species with an even spore septation were probably incorrectly classified. In this investigation, although one and three septate spores commonly occurred, a large number examined were of even septation.

The mature fructification in *S. pisi* has also been subject to critical review. Stevens (1925) stated that in *Phleospora* the pycnidia were poorly developed, and that many species of *Septoria* in early stages of development of pycnidia, would be included in *Phleospora*. Wallbroth, following Fries (Sprague, 1944), described *Phleospora* as having imperfectly formed pycnidia with fusiform bacillar, two to many septate spores.

This investigation has demonstrated the pycnidia to have a thin incomplete ill-defined wall, composed of loosely interwoven hyphae with

* The term "septum" is used in this paper with its meaning modified as indicated above.

no definite ostiole. These facts, together with the occurrence of spores of even numbered septation, suggest that the fungus identified as *S. pisi* may more correctly belong to the genus *Phleospora*. Of interest in this regard is Potebnia's (1910) observation that although he accepted the pathogenic fungus as *S. pisi*, it corresponded in development to the type species *Phleospora ulmi* (Fr.) Wallr.

Stevens (1925) stated that the genera *Septoria* and *Rhabdospora* were distinguished by the part of the host affected, leaf or stem respectively. The fungus discussed here was isolated from pycnidia on both stems and leaves, a fact in accord with the view of other authors, that *S. pisi* is identical with *Rhabdospora hortensis* Sacc.

Although the fungus described in this paper differs in certain controversial characters from other members of the genus *Septoria*, these differences do not seem to warrant another genus. The following description of the pathogenic fungus is given.

Septoria pisi Westendorp in Bulletin Academy Royal Society Belgium ser. ii, Vol. XII, No. 7, 1861.

Syns: *Septoria leguminum* Desmazières, Kickx. Fl. Fland., p. 424, 1867.

Rhabdospora hortensis Saccardo Mich. II, p. 103, 1880.

Lesions light brown, amphigenous, broad, irregular, elongated and limited by veins of leaves. Lesions also on stems. Mycelia inter- and intra-cellular.

Pycnidia straw-coloured, then black, scattered, 79.0-237.0μ diameter (av. 124.8μ), wall thin, of loosely interwoven hyphae, ostiole ill-defined. Spores cylindrical, straight or irregularly curved, hyaline, eguttulate, 24.5-59.0 $\times$ 3.0-3.5μ, 1-3 septate, issuing as yellowish spore horns.

Type locality: Belgium.

Distribution: Russia, Europe, North America, Australia and New Zealand.

Habitat: On Foliage and stems of *Pisum sativum* L. throughout New Zealand.

ACKNOWLEDGMENT

The author wishes to record his indebtedness to Dr. I. D. Blair, of Canterbury Agricultural College, under whose direction this investigation was carried out.

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A NOTE ON SEX RATIOS IN A ROMNEY MARSH STUD FLOCK

By R. A. BARTON, Massey Agricultural College, Palmerston North

(*Received for publication, 5 February 1948*)

In the course of an investigation into fertility in a flock of Romney Marsh stud ewes (Barton, 1947) data pertaining to sex ratios were secured, but were not published.

Most investigations into sex ratios of animals have been based on the ratio at birth, normally known as the secondary ratio. In the present data, the tertiary ratio, i.e., the sex ratio at maturity, as defined by Henning (1939), could not be ascertained because no complete record to maturity was kept for individual offspring. Even the secondary ratio in this study may be biased, as the sex was not recorded in the case of a small number of dead lambs.

Of the total number of lambs born (2,312), for which the sex was recorded, 49.7 $\pm$ 0.01 per cent were males. This figure agrees closely with that given by Chapman and Lush (1932), viz. 49.5 per cent (91,640 births) and that of Henning (1939) of 49.0 per cent (127,587 births).

The sex ratio of twin births in sheep has always attracted attention because a significant deviation from the binomial square suggests the occurrence of monozygotic twins in the flock. However, the investigations of Clark (1931), Johansson (1932), Chapman and Lush (1932) and Dry (1936) have not demonstrated the presence of identical twins in sheep, although Asheton (1898) and Henning (1937) have observed on post mortem rare and isolated cases of twins in utero where the ovary contained only one corpeus luteum.

The basis of calculation, as in most other studies attempting to establish the occurrence of monozygotic twins in sheep, has been the expected approximation of 1 ♂ ♂ : 2 ♂ ♀ : 1 ♀ ♀. It is assumed that this ratio is not materially changed by selective pre-natal mortality.

TABLE I. ACTUAL AND EXPECTED NUMBERS OF TWINS OF EACH SEX COMBINATION

Kind of Twin.	Observed.	Calculated.	Deviation.
Both males ♂ ♂	164	167·2	—3·2
Male ♂ and female ♀	340	338·5	+1·5
Both females ♀ ♀	173	171·3	+1·7

Table I shows the actual number and kind of twins in the flock under investigation. The expected frequencies have been calculated according to the formula $N(0.497 + 0.503)^2$, where N = total number of twin pairs and 0.497 = the proportion of all male lambs born to all female lambs born.

If monozygotic twinning occurred with any noticeable frequency, it would be expected that sets of twins of the same sex would be in excess of the male-female twin pairs. However, it is seen that there are only very small deviations between the observed and calculated figures and the χ^2 test gives a non-significant result.

As there were only eleven sets of triplet births recorded in this flock no valid conclusions can be derived from them. Actually the sex combinations were 2 ♂ ♂ ♂ : 4 ♂ ♂ ♀ : 4 ♂ ♀ ♀ : 1 ♀ ♀ ♀ which follows the same relationship as seen for twin sex combinations.

The above result can only serve to re-emphasize that monozygotic twinning is at most a comparatively rare phenomenon in sheep.

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DETERMINATION OF MERCURY ON WHEAT TREATED WITH AN ORGANIC MERCURIAL SEED DISINFECTANT

By H. J. McCoach, Dominion Laboratory, Department of Scientific and Industrial Research, Wellington

(*Received for publication, 9 May 1949*)

Summary

A method is described for the determination of mercury on wheat treated with an organic mercurial seed disinfectant.

This method gives good results and is suitable for checking treated wheat.

Introduction

The method described in this article was developed to determine the amount of mercury seed dust present on seed wheat that had been treated with an organic mercurial seed disinfectant, Ceresan.

A search of the literature revealed two possible methods, and these were investigated.

In the method of Strafford and Wyatt (1) the wheat is decomposed with hydrogen peroxide and sulphuric acid in a closed system and the mercury coprecipitated with copper by hydrogen sulphide. The sulphides and filter paper containing them are decomposed with sulphuric and nitric acids and the resulting solution is electrolysed with platinum electrodes. The copper and mercury are redissolved and the mercury estimated colorimetrically with p-dimethyl-amino-benzal-rhodanine.

Preliminary trials with this method showed several disadvantages. The decomposition of the wheat took far longer than was expected; the cathode became black during electrolysis and was frequently coated with a fine white fur-like precipitate; the colour produced by standard mercury solutions with p-dimethyl-amino-benzal-rhodanine was brick red whereas the colour produced by the test solutions was distinctly orange, making accurate comparison impossible; and lastly the recovery of mercury was only about 30-50 per cent.

The method was then checked by the addition of standard mercuric nitrate solution at various stages to find where the loss of mercury occurred.

When a mixture of sulphuric and nitric acids and copper and mercuric nitrates in the proportions met with in the method was electrolysed, full recovery of mercury was obtained, and it was noted that the cathode was evenly coated with a bright copper coloured deposit.

No loss of mercury occurred when copper and mercuric nitrates were heated with sulphuric and nitric acids, but losses of up to 50 per cent occurred when filter paper was included in the mixture. This loss was overcome by digesting the sulphides and filter paper with nitric acid at 300°C. in a sealed glass tube. Using this modification full recovery of mercury was obtained from the hydrogen sulphide precipitation stage and in further tests this method of decomposition of the sulphides was adopted.

When mercury was added to a solution of decomposed untreated wheat just before hydrogen sulphide precipitation the result obtained was of the order of 80 per cent of the amount of mercury added.

The recovery of mercury added to untreated wheat before decomposition, however, was only about 50 per cent.

This percentage recovery could not be improved upon and the method was therefore abandoned.

The second method investigated was that of Kozelka (2).

In this method the organic matter(blood and tissues) is decomposed with sulphuric acid and the mercury distilled off in a current of chlorine and estimated colorimetrically with dithizone.

Tests showed that wheat could not be digested satisfactorily with sulphuric acid alone, and this digestion was carried out with sulphuric and nitric acids in sealed glass tubes. The chlorine distillation was omitted owing to the absence of interfering metals in wheat and the mercury estimation was carried out directly on the digest from the sealed tubes.

Reagents and Apparatus

Sulphuric acid, concentrated, Analar.

Nitric acid, concentrated, Analar.

Ammonium hydroxide, sp.gr. 0.880, Analar.

Ammonium hydroxide, 9N.

Thymol blue indicator.

Dithizone solution, approximately 25 mg. of purified dithizone per 100 ml. of carbon tetrachloride.

Beckmann spectrophotometer.

Pyrex tubing, external diameter 25 mm., internal diameter 19 mm., made into tubes about 32 cm. long. A tube may be used for three or four decompositions, after which it becomes too short for further use.

A metal block, fitted with a pyrometer, in which the tubes are heated. The block is bored just deep enough to ensure that when the tubes are inserted the contents are wholly within the block.

The danger of an explosion is always present in work with sealed tubes and the heating of the tubes must be done behind carefully placed screens, while screens and face shields and gloves should be used when handling the tubes.

Procedure

Two grams of mercury disinfected wheat are weighed on to a small piece of filter paper and placed in a pyrex digestion tube. Sulphuric acid (6 ml.) and nitric acid (9 ml.) are added and the open end of the tube drawn out into a thick-walled capillary and sealed.

The tube is then heated gently in the metal block so that the temperature rises to 150°C. in half an hour. It is then allowed to cool to room temperature in the block. The tube is removed and the contents cooled for ten minutes in a freezing mixture of dry ice and alcohol. Any liquid in the capillary is driven into the body of the tube by gentle warming and the end of the capillary opened in an oxygen-gas flame. The tube is allowed to warm up to room temperature and as much as possible of the nitrous fumes is removed by shaking the tube in hot water.

The capillary is resealed, the tube heated to 250°C. in half an hour and held at this temperature for two hours. The tube is cooled and

opened as before and the top removed just below the shoulder. A fine stream of air is blown through the solution until brown fumes cease to be evolved, leaving the solution yellow or colourless. The solution is washed into a beaker and adjusted to about pH 1 by adding concentrated ammonium hydroxide until the indicator, thymol blue, just changes from red to yellow, care being taken to keep the solution from overheating.

The solution is cooled to room temperature, transferred to a separating funnel, and shaken for one minute with exactly 25 ml. of the dithizone—carbon–tetrachloride solution. The carbon–tetrachloride layer containing the mercury dithizonate is washed three times by shaking for one minute each time with 50 ml. of 9N ammonium hydroxide to remove excess dithizone. Quantitative transfer of the carbon tetrachloride is not necessary in each operation since the mercury is uniformly distributed in the 25 ml. of carbon tetrachloride originally added. The carbon tetrachloride is filtered through a filter paper to remove moisture and the optical density measured against a reference solution at the wavelength of maximum absorption of mercury dithizonate, viz. 4900A.°

The reference solution is made by treating 25 ml. of the dithizone—carbon–tetrachloride solution in the same manner and at the same time as the carbon–tetrachloride extract of the test solution, and two reference solutions are prepared each time.

A standard curve obtained by extracting as above aqueous solutions of mercury nitrate with 25 ml. of the dithizone solution and by plotting optical density against micrograms of mercury is a straight line for amounts of mercury from 0 to 100 μg.

It is essential that the same reagents be used throughout the method.

Results

The blanks from a number of samples of untreated wheat of different varieties from different sources are given in Table I, which shows that, using the same reagents each time, the blank is small and almost independent of the variety of wheat. The average value is 3.5 μg. of mercury in two grams of wheat, and this value was used in correcting subsequent determinations.

Recoveries of known amounts of mercury, added as standard mercuric nitrate solution to samples of untreated wheat, are given in Table II, which shows that all the mercury present is recoverable by the method.

The percentage of mercury in a sample of Ceresan was then determined by adding known amounts to untreated wheat. The average percentage of mercury from twelve determinations was 1.43, the mean difference from this value being 0.06 per cent of mercury.

The amount of mercury on the unknown samples for which the method was worked out was about 40 μg. on a two-gram sample, equivalent to about 1.2 oz. of Ceresan per bushel of wheat. With amounts of this order the method shows accurately whether or not the wheat has been properly dusted.

TABLE I.

Wheat Sample.	Blank, micrograms of mercury from duplicate determinations on two grams of wheat.
1	3.5
2	3.0
3	3.0
4	4.0
5	3.5
6	3.0

Average blank 3.5 μg. of mercury.

TABLE II.

Mercury Added. (μg.)	Mercury Recovered. (μg.)
19.9	18.5
41.3	41.0
61.2	61.5
81.1	81.5

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THYROID WEIGHTS OF CATTLE

By I. L. Campbell,* M. G. Hollard,† and D. S. Flux, The Dairy Research Institute (N.Z.), Palmerston North

(*Received for publication, 1 September 1949*)

Summary

(1) The mean weights and standard deviations of the thyroid glands of several classes of cattle in the Manawatu and Dannevirke districts have been determined and the mean weights have been compared with those published by overseas workers.

(2) A more detailed examination has been made of the weights of calf thyroids collected in the Manawatu in 1947. No significant difference between the mean thyroid weights of males and females was found. There was, however, a highly significant difference between the mean thyroid weights of calves from different collection districts.

Introduction

Data on the weights of the thyroid glands of animals may be of assistance in detecting abnormal thyroid glands, or may be used as a standard with which to compare the glands of experimental animals.

As little information on the weights of the thyroid glands of New Zealand cattle is available, data collected by the authors from several types of cattle slaughtered in the Manawatu district are presented.

Review of Literature

Krizenecky (1932) reported data from the work of Trautman (1917) showing thyroid weights of dairy cattle ranging from 23 to 41 g., and those of beef cattle from 21 to 36 g. The same writer quoted data from the work of Krupski (1921) from which the following table has been extracted:—

Table I. Thyroid Weight in Cattle—(Krupski 1921)

Type of Animal.	No. of Animals.	Age in Years.	Absolute Weight. (g.)
Beef cattle	40	1 - 5	21·49
Beef cows (pregnant)	21	1 - 4	21·76
Steers	67	1 - 5	42·08
Milk cows	171	2 - 13	32·59
Milk cows (pregnant)	69	3 - 11	31·80

No distinction was made between breeds within the dairy cattle or beef cattle groups, but Krizenecky stated that dairy cattle have larger thyroids than beef cattle.

Swett *et al.* (1937) compared the thyroid weights and body weights of four breeds of dairy cattle. Their data are summarized in Table II.

* Now Massey Agricultural College, Palmerston North.
† Now Canterbury Agricultural College, Lincoln.

TABLE II. AVERAGE THYROID WEIGHTS OF DAIRY COWS COMPARED WITH AVERAGE BODY WEIGHTS. (SWEET, MATHEWS, MILLER AND GRAVES, 1937)

Breed.	No. of Animals.	Live Weight. (24 to 30 hrs. after feeding.) (lb.)	Thyroid Weight. (g.)
Ayrshires	44	1,083	33·4
Guernseys	62	991	31·6
Holsteins	198	1,265	31·8
Jerseys	218	910	27·9

MATERIALS AND METHODS

The thyroid glands were obtained from animals slaughtered in the Palmerston North abattoirs and the freezing works at Longburn and Feilding. Calf thyroids were collected in late August and early September in 1946 and 1947.

In the latter year, thyroids from six districts were weighed separately. The glands from dairy cows were collected from February to October, those from vealers in February and March, and those from the steers, beef heifers, and beef cows, between January and March. Only calves which appeared to be wholly or predominantly of the Jersey breed were used, and as the glands were gathered during that part of the season when calves were collected from farms twice weekly, the great majority of the animals were two or three days old. The dairy cows which were dry or almost dry, and over two years of age were, for the most part, animals which had been discarded from dairy herds in the Manawatu district. The beef heifers were 2½ to 5 years of age. (At freezing works, the term "heifer" is applied to any female which has not been in milk.) In age and in breed the steers were a mixed group. Some had been fattened in the Hunterville district and some in the Kairanga, but they were not necessarily raised in these districts.

When the calves throats were cut and the animals bled, the larynx with a portion of the trachea with the thyroid attached was removed. The glands were taken to the Dairy Research Institute and kept at a temperature of 15°F. until they could be trimmed and weighed on a balance accurate to 0.1 g. Mature animals were stunned and bled, and the glands were removed from the tracheas, trimmed and weighed immediately on the same balance as the calf thyroids.

RESULTS

The mean weights and the standard deviations of the weights of the thyroids of the different types of animals are presented in Table III.

The weights of the calf thyroid glands collected in 1947 were recorded according to sex and the districts from which the calves came as shown in Table IV, and an analysis of variation is given in Table V.

TABLE III. MEAN WEIGHTS AND STANDARD DEVIATIONS OF BOVINE THYROIDS

Type of Animal.	Approximate Age.	No. in Group.	Mean Thyroid Weight. (g.)	Standard Deviation. (g.)
Dairy Calves.				
Jerseys, 1947				
Males	2 - 3 days	409	6·6	2·30
Females	2 - 3 ,,	225	6·5	2·31
Jerseys, 1946				
Males	2 - 3 ,,	33	7·4	2·39
Females	2 - 3 ,,	42	7·6	2·45
Dairy Cows.				
(Dry or almost dry)				
Jersey	2 years and over	81	29·5	8·64
Ayrshire		20	34·2	5·69
Friesian		8	42·0	12·60
Beef Vealers.				
Aberdeen Angus	6 - 9 months			
Males (castrated)		17	16·4	3·20
Females		7	17·6	5·32
Beef Heifers.				
Aberdeen Angus	2½ - 5 years	36	27·7	7·56
Beef Cows.				
Aberdeen Angus	3 years and over	15	31·8	4·51
Steers.				
Aberdeen Angus and Aberdeen Angus/ Hereford (cross)	2 years and over	87	29·1	7·29

TABLE IV. MEAN WEIGHTS OF THYROIDS FROM DIFFERENT DISTRICTS AND OF SEXES WITHIN DISTRICTS

Name of District.	No. of Calves.		Mean Weight of Thyroids. (g.)	Mean Weights of District Samples. (g.)	Unweighted Means. (g.)
Bunnythorpe	Males	70	7·2	6·9	6·6
	Females	30	6·0		
Shannon	Males	97	6·7	6·7	6·7
	Females	95	6·8		
Rangiotu	Males	45	5·4	5·6	5·7
	Females	31	6·0		
Rongotea	Males	82	6·9	6·9	6·8
	Females	35	6·7		
Kairanga	Males	24	6·0	6·5	6·8
	Females	10	7·6		
Dannevirke	Males	91	6·6	6·4	6·2
	Females	24	5·8		
TOTAL	Males	409	6·6	6·6	6·6
	Females	225	6·5		

TABLE V. ANALYSIS OF VARIATION OF WEIGHTS OF 1947 CALF THYROIDS

Source of Variation	D.F.	S.S.	M.S.
Districts	5	80·3168	16·06**
Sexes	1	0·036	0·04
Interaction	5	66·5963	13·32*
Individuals	622		5·09

** Significant at 1 per cent. level.
* Significant at 5 per cent. level.

DISCUSSION

There is considerable evidence indicating that thyroid size is influenced by iodine intake (Orr and Leitch 1929, Levine *et al.* 1933, Remington and Levine 1936, Remington 1937, Griffeth *et al.* 1939, McLendon and Foster 1947, Selye 1948). It is appropriate therefore to outline the information available on the iodine status of the soils of the districts concerned.

Rangiotu calf district lies mainly on young immature sand soils (Himatangi and Wananaki sands) in a coastal area of the Manawatu. Rongotea and Shannon calf districts include smaller proportions of the same soil types also adjacent to the coast. The remainder of these areas and the Kairanga, Bunnythorpe and Dannevirke districts lie on a variety of soil types, mostly alluvial. No systematic examination of the iodine status of soils or pastures in the areas covered by these calf districts has been made, with the exception of an area in and close round Palmerston North (in the Kairanga district) examined by Shore and Andrew (1934). These workers found that the iodine content of samples of soil taken by them in two surveys was uniformly low. However, the iodine content of vegetables grown in this area was not found to be below that of vegetables grown in areas where the soil iodine content was higher. Differences in the availability of soil iodine may be responsible for such a result (Leitch and Orr 1929, Simpson 1930, Hercus *et al.* 1931) although Greenwald (1946) has pointed out that different varieties of the same vegetables may differ in iodine content even when grown in the same soil and sampled at the same time.

Waters (1939-40) on the basis of low iodine percentages in the thyroids of lambs which he analysed, described the Manawatu-Wanganui district as being low in iodine, although he found no hyperplastic glands among his samples from this area.

The mean thyroid weights of the Jersey and Ayrshire cows are similar to those of Swett *et al.* (1937) (Table II), and of the same order as those for milk cows given by Krupski (1921) (Table I).

The mean weight of thyroids collected from calves in the year 1946 was significantly greater than that of the 1947 calves. It appears that some factor or factors influencing thyroid size may vary in effect from year to year. A similar yearly variation has been noticed in the weights of the thyroids of 2-3 day old calves killed on the Institute Experimental Farm, and in the weights of thyroids from lambs born on the Sheep Nutrition Area at Massey College, Palmerston North (Clarke 1949).

No significant difference between the weights of male and female calf thyroids was found in 1946 or in 1947 (Table V). Sykes (1935) obtained a similar result in young lambs.

The significant mean square for districts in Table V was caused by the lighter weights of the Rangiotu calf thyroids. The following Table shows the incidence of large and small thyroids from the different districts. Large thyroids have been taken as those heavier than the general mean plus the standard deviation, i.e. 6.57 g. + 2.27 g., and small thyroids, those lighter than the general mean minus the standard deviation, i.e. 6.57 g. – 2.27 g.

TABLE VI. FREQUENCY OF LARGE AND SMALL THYROIDS

District.	No. of Thryoids.	Large Thyroids.		Small Thyroids.	
		No.	Per cent.	No.	Per cent.
Bunnythorpe	100	9	9·00	6	6·00
Shannon	192	17	8·85	5	2·60
Rangiotu	76	3	3·95	16	21·05
Rongotea	117	16	13·68	7	5·98
Kairanga	34	6	17·65	6	17·65
Dannevirke	115	9	7·83	7	6·09
TOTAL	634	60		47	

Thyroids from the Rangiotu district were not only lighter on the average than those from the other bobby calf districts, but there were also fewer large and more small thyroids.

From the data available no reason can be given for the lighter weights of the thyroids from Rangiotu calves. Differences in the sizes and ages of the calves are not likely to have been responsible for this result, but cannot altogether be excluded as possible causes.

There is no apparent reason why the thyroids of male calves should in some districts be heavier and in other districts lighter than those of females of the same age. It is probable that the significant interaction between sex and pool in Table V is caused by chance errors in sampling.

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COPPER DEFICIENCY OF ONIONS GROWN ON PEAT. II.

By A. F. R. ADAMS, Canterbury Agricultural College, Lincoln, New Zealand

(*Received for publication, 10 July 1949*)

Summary

An account is given of a field trial with onions suffering from copper deficiency. Three methods of control all gave highly significant increases in crop yields. The greatest yields were obtained from the application of a foliar spray of 0.25 per cent copper sulphate in water, twice during the growing season.

Broadcast applications of powdered copper sulphate are likely to be favoured in practice.

INTRODUCTION

IN a previous paper (1), an account was given of a condition found in onions, wherein the plants failed to make normal growth and did not mature. The area affected is a peat soil in the vegetable growing area of Marshland (near Christchurch) and it was found that the application of a 0.25 per cent solution of copper sulphate as a foliar spray gave a marked increase in the leaf, bulb, and root, growth. The close similarity of the symptoms to those noted by other workers, together with the very striking response to treatment with copper sulphate, pointed to a deficiency of copper available to the plant. It was therefore decided to conduct a further trial to determine the relative effectiveness of three methods of application of copper sulphate.

A survey of the district has shown that this disorder reaches serious proportions in only a small area of the peat land under cultivation, but appears to be present elsewhere in the district in isolated pockets and in a much less severe form. It was also noted that even in those areas severely affected, there was often a marked variation in the degree of attack within a small area.

EXPERIMENTAL

The trial took the form of six replicates of four treatments in randomized plots each five yards by three yards, laid out in a strip across the field. Prior to sowing the trial area received one ton limestone per acre and 10 cwt superphosphate per acre.

The four treatments were:—

A. Control—No added copper;

B. Two foliar sprays of 0.25 per cent copper sulphate ($CuSO_4.5H_2O$), in water with a wetting agent, each equivalent to 20 lb. copper sulphate per acre;

C. One heavy soil dressing of powdered copper sulphate, at 200 lb. per acre, broadcast prior to sowing;

D. One light soil dressing of powdered copper sulphate at 56 lb. per acre broadcast prior to sowing.

The fertilizers were worked into the topsoil by means of hand raking. The seed was sown in mid-September with a Planet Junior drill in rows approximately 13 in. apart, and the seedlings were showing by the end of that month. A germination count carried out at any early stage showed no significant differences between treatments.

The 'B' plots received their first spray application on the 23rd October 1947, when the plants were about two inches high. The solution was applied from a watering can along the rows of plants only. No deficiency symptoms were apparent on any plots when the area was inspected on the 10th November, by which time the plants had reached a height of four to five inches. By the 4th December, however, definite symptoms were apparent, viz., withered tips, leaves losing turgidity and bending back, particularly in the control plots 'A,' and to a lesser extent in the light soil dressing plots 'D.' The heavy soil dressing 'C' and spray treated plots 'B,' were indistinguishable to the eye; the plants showed no abnormality and presented a much more healthy appearance than those in the two other treatments. The 'B' plots received their second and final spray on the 12th December.

During the next five or six weeks the 'B' plots became noticeably stronger in growth than the 'C' plots, this difference appearing consistently in all replications. Also, the difference between the control and all other treatments became more and more striking, many of the plants in the control plots dying back until they finally shrivelled to a dead stem with no bulb at all (Fig. 1). The 'D' plots were better than control 'A' but still very poor and very much inferior to the 'B' and 'C' plots.

CROP YIELDS

In keeping with the usual practice of growers in the district, the onions were not thinned as seedlings. The friable nature of the top soil is such that the bulbs displace each other sideways during growth with no detrimental effect on shape or size. The crop was pulled when all the tops had died back (23rd February) and the bulbs allowed to lie on top of the soil to ripen. To minimize possible border effects, one foot from each end and the two outside rows on either side of each plot were discarded, leaving nine seven-foot rows per plot to be harvested. The onions were topped after ripening and the individual plot yields of bulbs recorded. These are shown in Table I.

FIG. 1.—Copper deficiency of onions. January 1948. Left: Treatment B, (Spray); Right: Control.

TABLE I. YIELDS OF BULBS IN POUNDS

Treatment.	Replicates. 1	2	3	4	5	6	Mean.
A	3·9	5·1	3·8	3·0	0·6	3·4*	3.3
B	37·9	35·6	36·6	20·4	15·3	26·1	28·7
C	24·8	21·6	21·1	12·0	8·9	11·8	16·7
D	13·8	7·4	9·4	6·3	3·7	3·5	7·4

* Atypical plot.

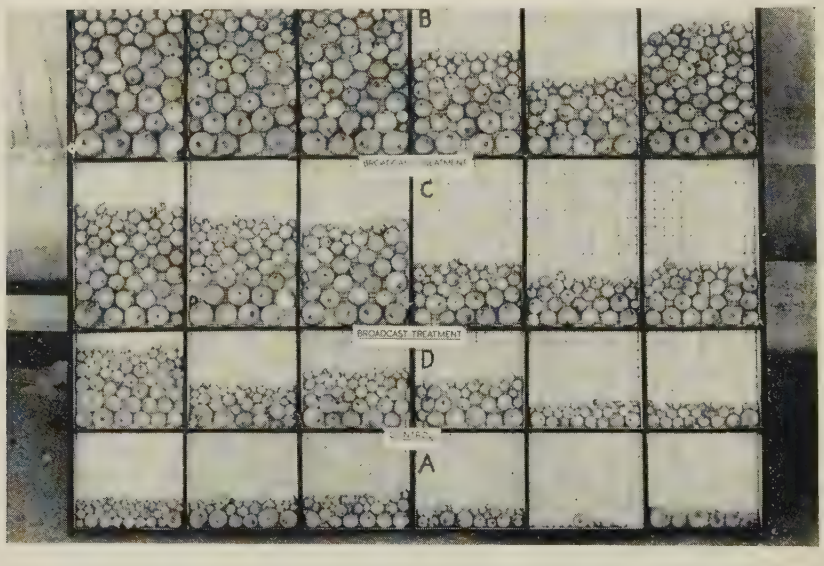

FIG. 2.—Representative samples of individual plot yields. Vertically: The four treatments; Horizontally: Replicates 1-6.

A representative sample (one-eighth) of the yield from each plot was photographed to show the yield and size increase brought about by the different treatments(Fig. 2).

It will be seen that there was a fertility gradient across the field with yields decreasing from replicate 1 to replicate 5, but rising again in replicate 6. This, however, did not affect the significance of the results.

Omitting the control plot in replicate 6 which was judged atypical because of the presence of one row of normal onions in an otherwise almost bare plot, the ratios of mean treatment yields (bulbs) from all plots were:—

A.	Control	100
B.	Sprayed	870
C.	Heavy soil dressing ..	510
D.	Light soil dressing ..	220

The whole crop was also graded through a 1½ in. square grid to obtain relative yields of onions of marketable size. This grading further accentuated treatment differences and gave the following relative marketable yields:—

A.	Control	100
B.	Sprayed	2,710
C.	Heavy soil dressing ..	1,290
D.	Light soil dressing ..	310

ANALYTICAL RESULTS

Soil:

Table II gives a typical analysis of the peat, sampled to four inches, on which this trial was conducted. The usual analytical methods were employed, organic carbon being estimated by oxidation with chromic acid.

Copper Content of Soil:

The total copper content was estimated by a wet digestion procedure along lines very similar to those employed by Clare *et al.* (2) and gave results varying from 7.5 to 9.2 p.p.m. on an oven dry basis, for different portions of the field. This indicated that the problem of this soil was one of availability of copper rather than a definite lack in the soil and an attempt was therefore made to assess the available copper by the bioassay method using *Aspergillus niger,* a method used with considerable success by Acock (3). A culture of the particular strain (M) known to be most suited to this work was kindly made available by the Biotics Section of the Soil Bureau. Using the technique outlined by Acock with 0.5 g. air dry soil, figures greater than 4 p.p.m. of copper (the upper limit of the test) were obtained for all soils tested. By using a smaller quantity of soil in an endeavour to bring this figure within the range covered by the test, it was estimated that the available copper was probably of the order of 5 - 6 p.p.m. Difficulty was experienced here, however, in obtaining a uniform spread of soil over the area of the petri dish. This figure (5 - 6 p.p.m.) is greater than that usually found in copper deficient soils, but confirms results obtained by the Soil Bureau for other known copper deficient soils of high organic matter content. (priv. comm.)

In this connection it is interesting to note the successful use of this method by Nicholas and Fielding (4), who obtained a figure of 0.5 - 0.75 p.p.m. of copper (indicating Cu deficiency) on an alkaline fen soil of high organic matter content known to be responsive to copper salts.

TABLE II. SOIL ANALYSIS

Dry matter basis (per cent.).							Air dry basis.								
				Soluble in 1 per cent. citric acid.			Base Exchange (per cent.).								
SiO_2.	Fe_2O_3 Al_2O_3 etc.	CaO.	MgO.	Loss on ignition.	K_2O.	P_2O_5.	pH.	Base Exch. Cap. m.e.	Total Exch. Bases m.e.	Base Saturation.	Exch. CaO m.e.	Exch. MgO m.e.	Exch. K_2O m.e.	Org. C.	Tot. N.
15·2	2·1	1·45	0·14	77·0	0·034	0·019	4·8	116·0	60·1	51·8	55·5	6·5	0·8	47·4	2·12

TABLE III. ANALYSIS OF ONIONS

Copper Content of Onion Bulbs and Tops (p.p.m. on moisture-free basis).

Treatment.	Bulbs.				Tops.			
	A.	B.	C.	D.	A.	B.	C.	D.
Replicate 1	2·6	2·2	4·3	2·4	2·8	5·8	14·6	8·2
2	1·5	2·3	3·0	2·8	3·2	5·5	15·9	6·2
3	2·0	3·1	2·6	2·0	3·7	7·2	18·0	11·1
4	1·6	3·3	3·5	2·2	3·4	7·0	19·4	10·2
5	1·3	3·2	2·3	1·7	2·6	5·7	31·3	8·2
6	1·8	3·2	2·7	2·3	2·2	3·5	18·1	10·1
Mean	1·8	2·9	3·1	2·2	3·0	5·8	19·4	9·0

DISCUSSION

Two points stand out from Table III:

(1) Although there has been an increase of from 22 - 72 per cent in the copper content of the bulbs, the highest figure is still low for normal onions according to results recorded in the literature.

(2) The highest yields were obtained from the sprayed plots 'B', yet the copper content of both bulbs and tops from these plots is lower than that from the plots receiving the heavy soil dressing 'C'.

Several workers in this field have stated that the need for copper is greatest in the early seedling stage of a plant's growth. Thus Rademacher (5) states that the copper content of oats is at its maximum at the three leaf stage (11.6 p.p.m.) decreasing steadily to a fairly constant level of ca. 3.4 p.p.m. He suggests that the period of maximum requirement is during early growth and that copper deficient soils do not supply copper fast enough to carry plants over this critical period. Piper (6) reached the same conclusion and stated that while applications of copper sulphate gave highly significant yield increases, the increased absorption of copper by plants so treated was surprisingly small. He quotes the copper content of oat plants at flowering as 1.22 p.p.m. in controls and 1.38 p.p.m. when treated with 1 cwt. of copper sulphate per acre giving a yield increase of 100 per cent. The same effect is noted by Riceman *et al.* (7).

It is probable, therefore, that in this trial the spray treatment supplied readily available copper during that early critical period of maximum need and, together with later spraying, enabled the plants to grow on without a check. Also, that owing to dry weather during the early growing period, the broadcast dressings did not prove as effective in supplying the much needed copper in the early stages, but with increasing root range enough copper was absorbed to sustain the plant and to overcome to a considerable extent the acute copper deficiency.

The improvized spray method of applying copper sulphate used in this trial would be impracticable and wasteful on a large scale, but there appears to be no reason why the same beneficial effect should not be obtained with a very much reduced volume of spray of the same solution (0.25 per cent) provided good coverage was ensured. This should be easily attained with good spray equipment. For the present, however, it would seem that the method likely to be favoured by most of the growers is broadcasting, and many growers adopted this procedure

during the season just completed. Another method of application used by some growers was drilling beneath the seed in combination with other fertilizers, but unless the fertilizer is placed well down, there appears to be a danger of damage to young seedlings, through the tender roots running into zones of high concentration of copper sulphate.

A probable benefit to be gained from broadcast dressings is a residual effect. Steenbjerg (8) stated that the residual effects of copper sulphate did not decrease in four years, and many other workers similarly claim a lasting effect. Evidence for such an effect in the area under discussion is the fact that on a block of approximately three acres which was treated with copper sulphate at 200 lb. per acre in August 1947, and which grew a good crop of onions that year, a heavier crop of very firm, well-coloured onions was obtained in the 1948-49 season with no further addition of copper.

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